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XERODERMA PIGMENTOSUM EPIDERMAL CELLS WITH NORMAL UV-INDUCED THYMIDINE INCORPORATION*

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ABSTRACT

Ultraviolet light-induced thymidine incorporation was measured in trypsin-dissociated epidermal cells from two patients with xeroderma pigmentosum (XP) and from control donors. Results paralleled those obtained with lymphocytes and fibroblasts in that the patient with a repair defect in these cells also had the defect in her epidermal cells. The other patient, previously shown to have no detectable repair defect in his lymphocytes or fibroblasts, appeared also to have normal DNA repair in his epidermal cells despite the presence of severe clinical manifestations of XP in this tissue.

Most patients with xeroderma pigmentosum (XP), a recessively inherited disease characterized by actinic damage and multiple cutaneous malignancies on sun-exposed areas, have a defect in the process by which UV-induced thymine dimers are removed from DNA. This excisional-repair process in XP has been evaluated by measuring the rate of dimer excision (1, 2) or the rate of UV-induced nucleoside uptake into DNA during repair synthesis through studies of unscheduled thymidine incorporation (3–13), repair replication (3, 14, 15), and bromodeoxyuridine photolysis (16, 17). Thus, cells from some XP patients have no detectable DNA repair (3, 5, 11, 14) while others have repair rates which are lower than normal (3–15). Recently, however, we reported an XP patient (designated patient 4 in our XP series) with a normal rate of UV-induced tritiated thymidine (^{3}HTdR) incorporation into his peripheral blood lymphocytes (8) and skin fibroblasts (9). Subsequent repair replication (18) and bromodeoxyuridine photolysis (19) experiments have confirmed that this patient's fibroblasts lack the repair defect present in cells from all previously studied XP patients. Cultured fibroblasts from the patient appear to have a normal degree of survival, as evidenced by colony formation following UV irradiation (18), suggesting that they have no defect in any aspect of DNA repair. Two additional patients, siblings, have more recently been reported to have normal DNA repair in their skin fibroblasts (18).

Since the epidermis is the primary site of clinical disease, including neoplasia, in XP, it was important to determine whether or not the epidermal cells of patient 4, like his peripheral blood lymphocytes and fibroblasts, had a normal rate of UV-induced ^{3}HTdR incorporation. In order to resolve this question, we have performed studies in which this patient's epidermal cells, dissociated by trypsin, were irradiated in vitro, incubated with ^{3}HTdR, and then studied radioautographically to evaluate their UV-induced ^{3}HTdR incorporation. In this paper we present the details of this method for studying UV-induced ^{3}HTdR incorporation into epidermal cells in vitro and the results obtained using epidermis from patient 4, from an XP patient who is known to have the repair defect in lymphocytes and fibroblasts, and from control donors who do not have XP.

MATERIALS AND METHODS

Subjects. The XP patients whose skin was used for these studies were patients 1 and 4 of our XP series.† Their clinical histories have already been briefly presented (8). Patient 4 has recently died of metastatic malignant melanoma, and a detailed clinical and pathologic case report is in preparation and will be presented elsewhere. Skin from three male Caucasian control donors was also used in the present study. Control donor A, 38 years old, is a fair-skinned Texan who has had numerous actinic keratoses and basal cell carcinomas following years of excessive exposure to sunlight. He has had no known squamous cell carcinomas and no evidence of basal cell nevus syndrome. Control donor B, 49 years old, has basal cell nevus syndrome (20), a disease in which there is no known defect in repair of UV-induced damage to DNA (21). Control donor C, 21 years old, is a healthy volunteer.

Preparation of epidermal cell suspensions. The techniques for obtaining dissociated epidermal cells were modified from methods used by other investigators (22). Skin on the lower back or anterior thigh was washed with 70% ethanol. A 2- to 3-inch-long strip of skin was then removed from one of these areas with a 1.3-inch-wide Davol keratotome set to cut at a depth of 0.015 inches. The skin strip was placed with its dermal surface upward in a Petri dish and incubated for 45 minutes at 38°C with an 0.3% solution (w/v) of trypsin 1:250 (Cat. No. 0152; Difco) dissolved in Medium 199 (containing bicarbonate, phenol red, penicillin and streptomycin). Prior to this incubation the pH of the solution was adjusted to approximately 7.6 with a 10% aqueous solution of sodium bicarbonate. After the 45-minute incubation, the strip of skin was rinsed in three

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† Skin fibroblast cell lines from all of our XP patients and from some of their parents are available from the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland 20852, U. S. A.

successive 10-ml portions of Medium 199, and it was then incubated for 15 minutes at 38°C in a solution composed of 6 parts of Medium 199 and 4 parts of human plasma obtained from heparinized blood in order to inactivate any residual trypsin. After this incubation, the skin was transferred to a dry Petri dish, and the dermis was easily peeled and removed from the epidermis with forceps. A piece of the epidermis was fixed in 10% formalin and processed histologically to permit microscopic examination of the epidermis for contamination with attached residual dermal tissue. The remaining epidermis was covered with Medium 199, and cut and scraped with a scalpel. The resulting tissue suspension was then gassed with a carbon dioxide–air mixture (5%:95%) and agitated on a Fisher rotator for 15 minutes. It was centrifuged in a 10-ml conical centrifuge tube at 150 *g* for 6 minutes, and the supernate was discarded. The resulting pellet was resuspended in approximately 10 ml of culture fluid composed of 4 parts of Medium 199 and 1 part of autologous plasma. After approximately 1 minute the large clumps of cells and keratinous debris had settled to the bottom of the tube, and the supernate, containing the dissociated epidermal cells, was obtained. The cell concentration in this resulting epidermal cell suspension was determined in a hemocytometer, and the suspension was diluted with additional culture fluid to a concentration of 0.5×10^6 epidermal cells per ml.

Irradiation of the epidermal cells and their incubation with ^{3}HTdR. Two-ml aliquots of the cell suspension were placed in each of two Petri dishes resulting in a cell suspension 1.0 mm deep in each dish. While the contents of both dishes were being gently shaken, the suspension of one was irradiated at room temperature with UV light at an incident flux of approximately 1 erg/mm^2/second for approximately 130 seconds from a 254 nm Germicidal lamp (General Electric lamp No. G15T8). Immediately after irradiation the irradiated and nonirradiated suspensions received ^{3}HTdR (thymidine-methyl-^{3}H; 22.4 or 26.0 Ci/mmole; Cat. No. TRK-120; Amersham–Searle) at a final concentration of 12.5 μCi/ml of cell suspension and were regassed. The suspensions were incubated in a water bath at 38.5°C for 3 hours after which they were removed and allowed to cool to room temperature. Forty-five minutes after their removal from the water bath the suspensions were centrifuged, and each of the two cell pellets was washed and resuspended 3 times in 2 ml of cell-free culture fluid. The cell pellets were dispersed in a few drops of cell-free culture fluid, smeared on coverslips, and air-dried.

Radioautography. The coverslips, with their cell-bearing surface upward, were attached with Permount to standard microscope slides which had previously been coated with an 0.5% gelatin solution (23) to promote adherence of the radioautographic emulsion. After the Permount had hardened, the cells were fixed by two 5-minute immersions of the slides in ethanol-acetic acid (3:1), washed by two 3-minute immersions in 70% ethanol, and air-dried. The radioautograms were made by coating the slides with NTB-3 emulsion (Eastman Kodak). After an exposure at 4°C for 2 or 4 weeks, the radioautograms were developed and stained with Wright's stain. The number of grains over the nucleus of each of the consecutively observed cells, isolated or in clusters of 3 or fewer cells, was determined by an observer who had no knowledge of the cell donor or irradiation treatment of the cells under observation.

RESULTS

The removal of the epidermis from the underlying dermis is illustrated in Figure 1. Figure 1a shows part of a dermatome shaving in which separation of dermis from epidermis was not complete. The dermis is separated from most of the epidermis in this section and is still attached in only one location. Areas with such incomplete separations were discarded. Figure 1b shows epidermis which has been completely separated from the underlying dermis. As Figure 1c shows, the separation has occurred along the dermo–epidermal junction. Dissociation of the cells in such epidermal sheets yielded epidermal cell suspensions in which the majority of cells occurred singly with the remainder in small clusters (Fig. 2).

Figures 3–5 show 4-week-exposed radioautograms of isolated epidermal cells from control donor B (Fig. 3), patient 4 (Fig. 4) and patient 1 (Fig. 5). To obtain visualization of the radioautographic grains, these photomicrographs were taken with the grains, rather than the cells, in focus. Nonirradiated cells which are not in semiconservative (S-phase) DNA synthesis show only an occasional grain over their nuclei (Figs. 3a, 4a, 5a). Cells in semiconservative DNA synthesis in preparation for mitotic division are "heavily labeled" (arrows). Irradiated epidermal cells from control donor B (Fig. 3b) and from patient 4 (Fig. 4b) have many grains which produce "lightly labeled" nuclei, thereby reflecting the UV-induced ^{3}HTdR incorporation which had occurred during repair replication. Irradiated cells from patient 1 (Fig. 5b), however, show much less UV-induced ^{3}HTdR incorporation.

The Table presents radioautographic data from nonirradiated epidermal cells from patients 1 and 4 and from each of the three control donors. The radioautograms were exposed for 2 weeks and then developed. Ninety-four percent of the cells had fewer than 5 grains per nucleus, and approximately 98 percent had 10 grains or fewer per nucleus.

Figure 6 shows radioautographic data from each of these subjects' UV-irradiated epidermal cells after the same 2-week radioautographic exposure. Each histogram depicts classes of the grain number per cell nucleus for 250 cells from each subject. Only 6.0 percent of the cells from patient 1 had more than 10 grains per nucleus in contrast to 39.6 percent of the cells from patient 4. The control donors, A, B, and C, had 44.8, 74.4, and 18.4 percent, respectively, of their cells labeled with more than 10 grains per nucleus. Thus, the number of such labeled cells from patient 4 was well within the range given by cells from the three control donors.

The histograms indicate also that the irradiated cells from patient 4 and from the three control donors are comprised of two distinct populations.

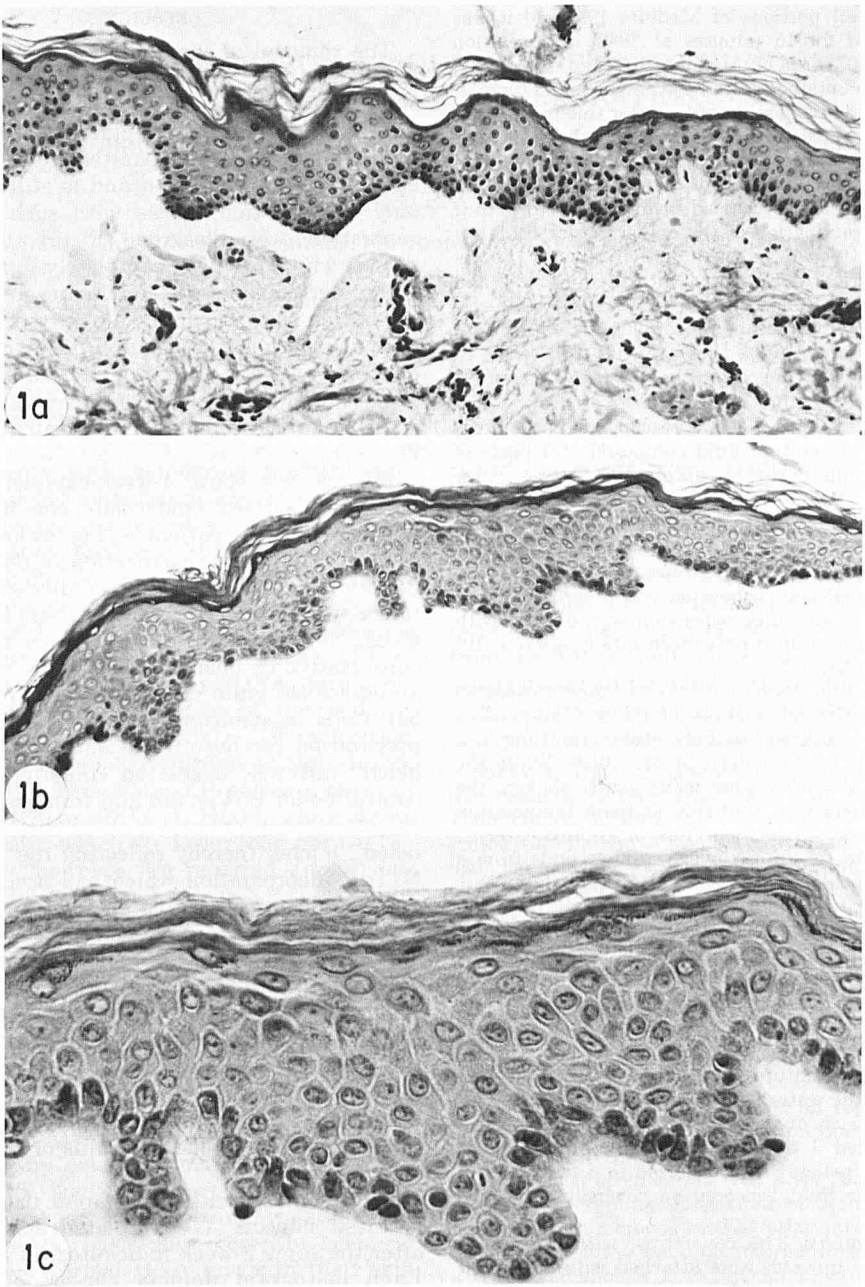

FIG. 1. Cross sections of trypsin-treated skin illustrating removal of dermis from epidermis. (a) Portion of a specimen in which the dermis was not completely removed from epidermis; × 160. (b) Specimen showing epidermis completely separated from dermis; × 160. (c) Higher-power view of previous specimen indicates no attached dermal contamination of epidermis; × 400. H & E.

One population, like the nonirradiated cells (Table), has 5 or fewer grains per cell nucleus and thus shows no evidence of UV-induced ^{3}HTdR incorporation; the other population, with more than 5 grains per cell nucleus, clearly evidences such incorporation. The mean grain number per cell nucleus for this labeled population (i.e., for all cells with more than 5 grains per cell nucleus) was calculated and is indicated by the arrows. Patient 4's mean grain number per cell nucleus was 36.0, a value well within the range of the values, 41.3, 33.7, and 34.5, given by control donors A, B, and C, respectively. The mean grain number per cell nucleus for the cells of patient 1 was 11.9, approximately one-third of the values given by the cells of the other subjects. Most of patient 1's detectable

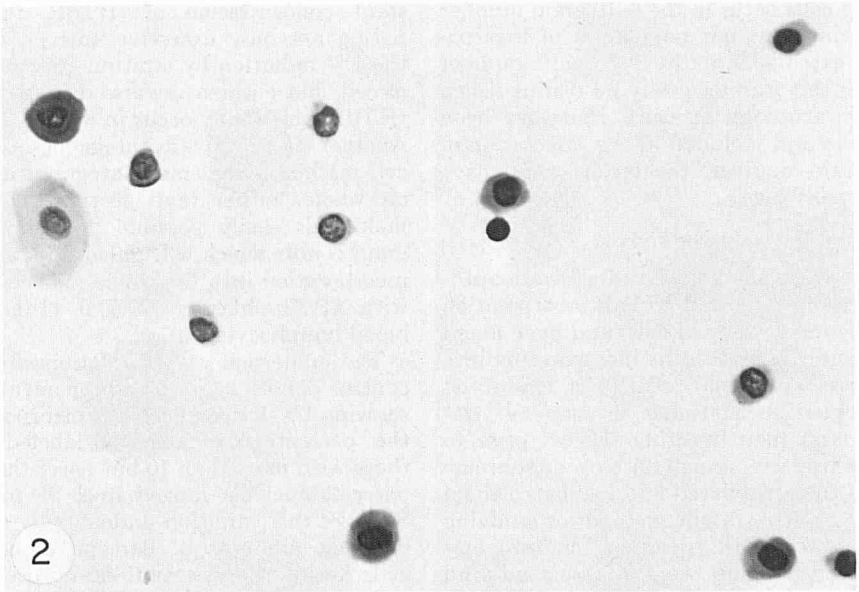

FIG. 2. Smear of an aliquot of dissociated epidermal cells after their suspension in culture fluid. Giemsa. × 400.

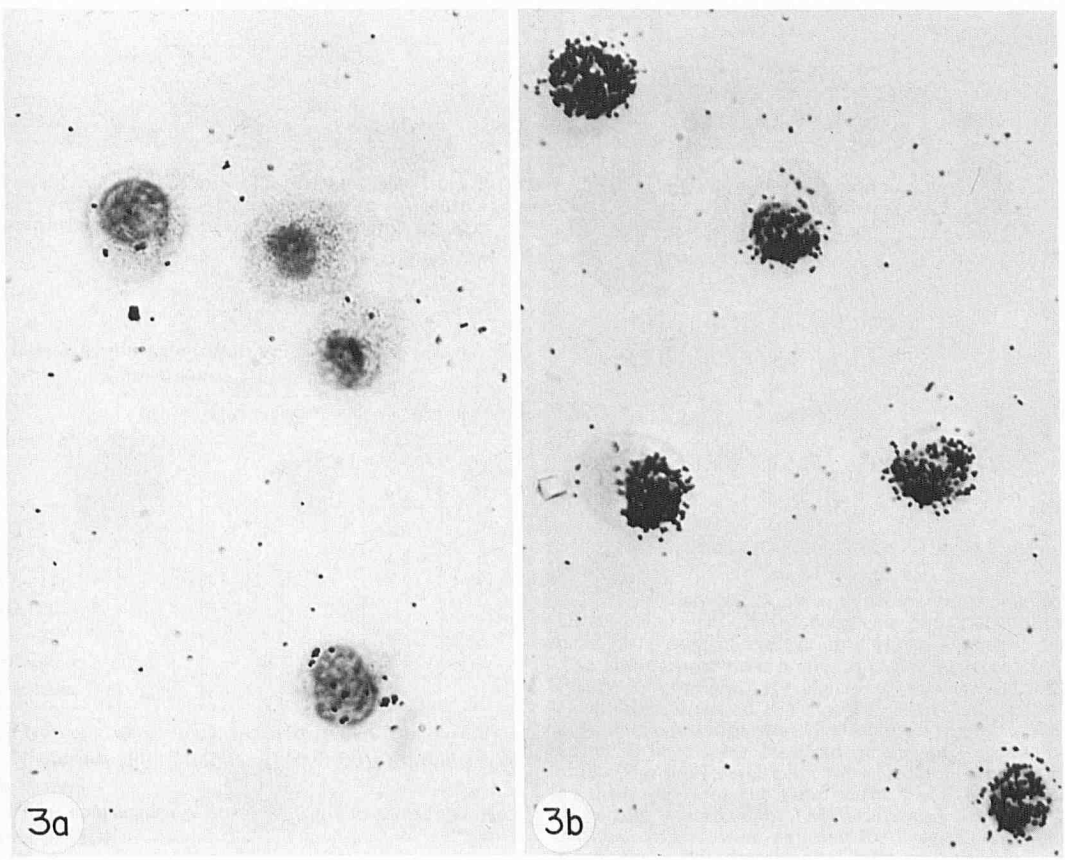

FIG. 3. Radioautograms of epidermal cells from control donor B. (a) Nonirradiated; (b) irradiated as described in "Materials and Methods." Radioautographic exposure, 4 weeks. Wright's stain. × 1000.

incorporating cells occur in the 6–10 grain number class, indicating that her population of incorporating cells extends into the 0–5 grain number class wherein they cannot easily be distinguished from the nonincorporating cells. Had they been distinguishable and included in the calculation of her mean grain number, the latter would have been considerably lower.

DISCUSSION

Other investigators have studied radioautographically the UV-induced ^{3}HTdR incorporation into XP patients' epidermal cells and have found it to be markedly less than the incorporation into normal subjects' epidermal cells. Thus, Epstein et al (5) performed such studies in vivo by irradiating skin and then injecting ^{3}HTdR prior to biopsy of the test site. Jung (10) took dermatome shavings and then irradiated and incubated them with ^{3}HTdR in vitro. In our method for studying the UV-induced ^{3}HTdR incorporation into epidermal cells, the cells were dissociated and placed in suspension prior to irradiation and incubation with ^{3}HTdR. Advantages of our technique are that every isolated cell in the suspension can be exposed to essentially the same radiation dose and can be bathed in the same, constant concentration of ^{3}HTdR, thereby eliminating not only excessive, uneven absorption of the UV radiation by stratum corneum and layers of cells but eliminating also diffusion gradients of ^{3}HTdR which may occur in intact epidermis (24). Another technical advantage of our dissociated cell method is that radioautograms are performed on whole, rather than sectioned, cells thereby making it easily possible to obtain meaningful grain counts which will reflect the level of ^{3}HTdR incorporation into the whole nucleus as was done with XP fibroblasts (3, 4, 7, 9, 11) and peripheral blood lymphocytes (8).

The epidermal cell populations from our three control donors had different percentages of cells showing UV-induced ^{3}HTdR incorporation. Thus, the percentage of "lightly labeled" cells (i.e., those with more than 10 but fewer than 100 grains per cell nucleus) ranged from 18 to 74 percent. Some of this variation undoubtedly resulted from different amounts of damage to each subject's cells during the dissociation and preparative procedures as well as from possible differences in the inherent levels of repair of these individuals. Thus, Epstein et al (25) have reported that the number of lightly labeled cells in UV-irradiated intact epidermis "varied greatly from person to

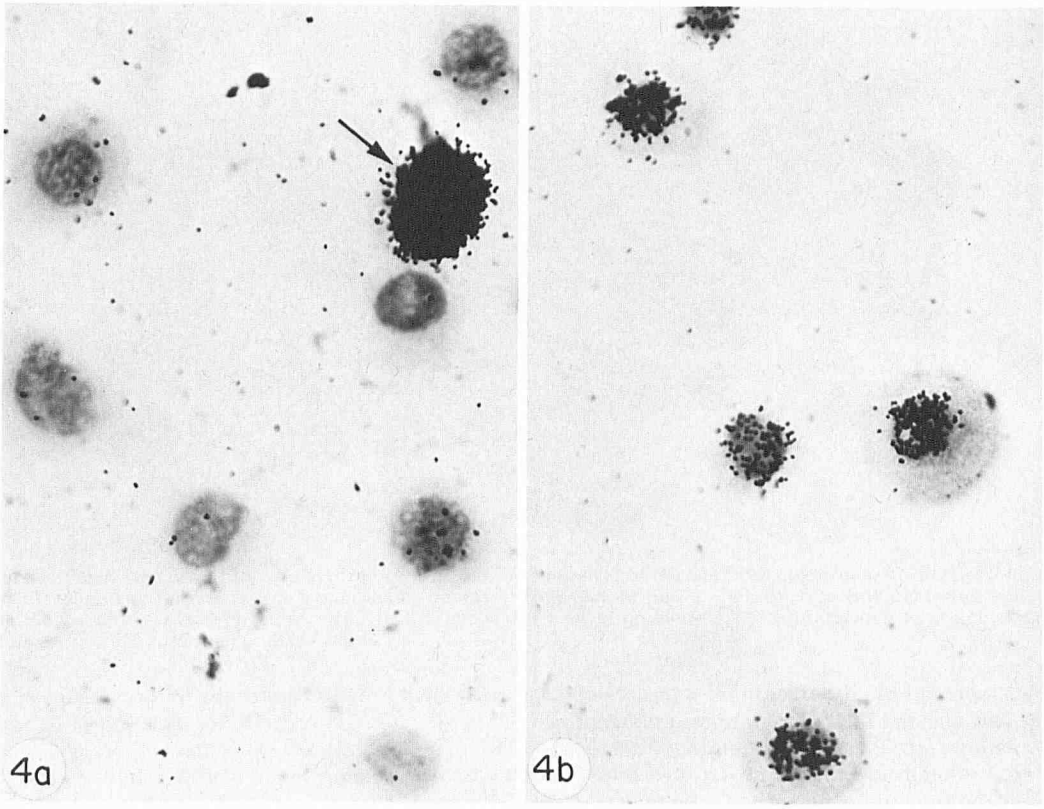

FIG. 4. Radioautograms of epidermal cells from patient 4. (a) Nonirradiated; (b) irradiated as described in "Materials and Methods." Radioautographic exposure, 4 weeks. The arrow indicate a "heavily labeled" cell in semiconservative DNA synthesis. Wright's stain. × 1000.

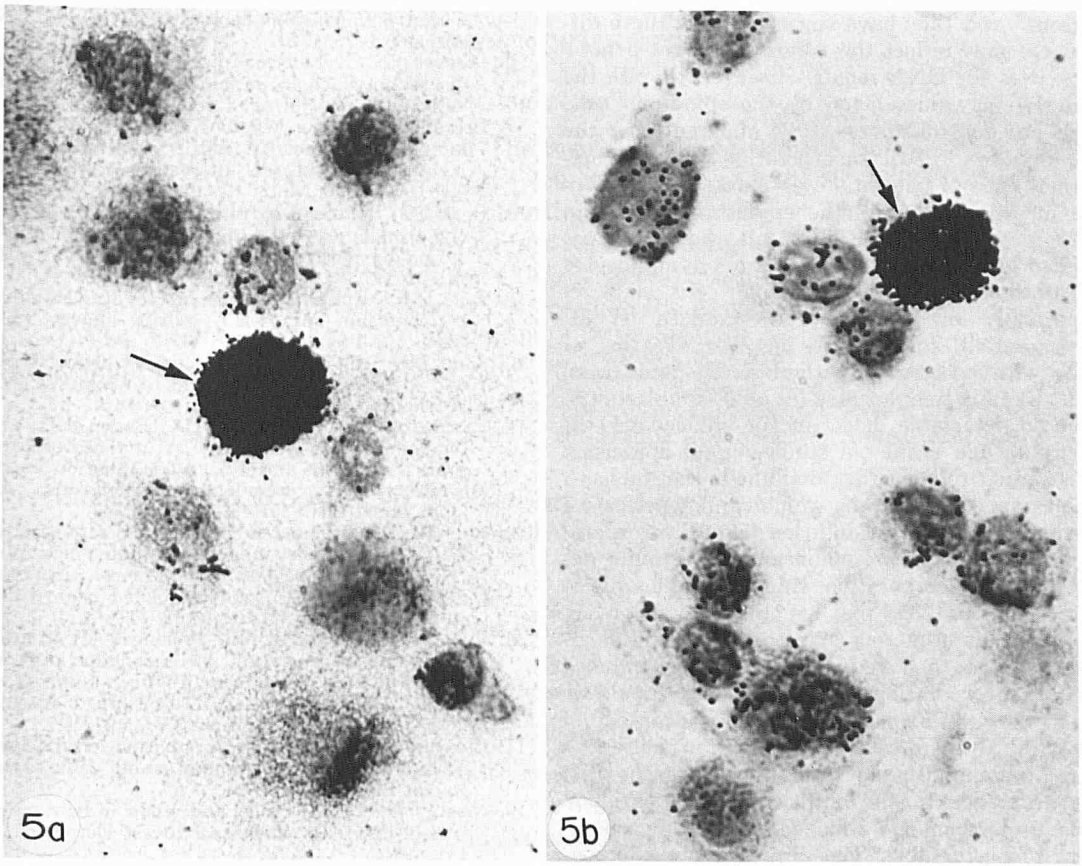

FIG. 5. Radioautograms of epidermal cells from patient 1. (a) Nonirradiated; (b) irradiated as described in "Materials and Methods." Radioautographic exposure, 4 weeks. The arrows indicate "heavily labeled" cells in semiconservative DNA synthesis. Wright's stain. × 1000.

TABLE

Radioautographic distribution of grains over nuclei of nonirradiated epidermal cells

Grains per cell nucleus*	Number of cells with the indicated grain numbers from:†				
	XP patient		Control donor		
	1	4	A	B	C
0–5	50	47	46	43	49
6–10		3	3	4	1
11–15				3	
16–20					
21–25					
26–30			1		
31–200‡					

* The cells were incubated with ^{3}HTdR as described in "Materials and Methods." Radioautographic exposure, 2 weeks.

† Fifty consecutively observed cells from each subject were analyzed.

‡ Nuclei with more than 200 grains represented cells in semiconservative DNA synthesis. Their frequency was approximately 1 per 250 cells.

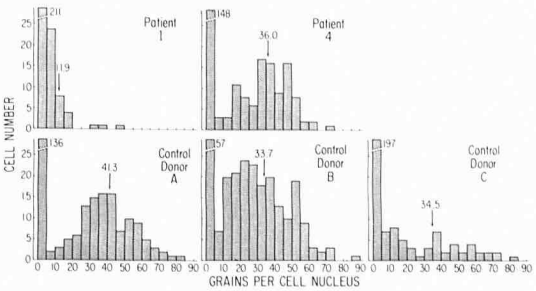

FIG. 6. Histograms showing radioautographic data of UV-induced ^{3}HTdR incorporation into 250 epidermal cells from each of the patients and the control donors. The 250 cells of each histogram were obtained by analyzing 50 consecutively observed cells on each of five coverslips except in the case of control donor C where only four coverslips were available; in the latter case an extra 50 cells were analyzed on one of the four coverslips. The epidermal cells were from the same batches as those used for the data of the Table but were irradiated as described in "Materials and Methods." The radioautograms were exposed for 2 weeks. Nuclei with more than 200 grains, i.e., cells in semiconservative DNA synthesis, were excluded. Each arrow indicates the mean grain number per cell nucleus for all of a subject's cells having more than 5 grains per nucleus.

person," and they have suggested that these differences may reflect the donors' different genetic capacities for DNA repair. However, despite the variation in values given by the epidermal cells from our control donors, it is apparent that the UV-induced ^{3}HTdR incorporation into the epidermal cells of patient 1 was markedly less than the incorporation into the epidermal cells from these control donors. Thus, all the cell types studied from this patient, i.e., her lymphocytes (8), fibroblasts (9), and epidermal cells, show an abnormally low amount of UV-induced ^{3}HTdR incorporation. In contrast, however, are the results with cells from patient 4. His epidermal cells, like his lymphocytes (8) and fibroblasts (9), have no detectable defect in UV-induced ^{3}HTdR incorporation. While our studies on his epidermal cells cannot rule out the possibilities that he has a small population of cells which cannot perform a normal amount of UV-induced ^{3}HTdR incorporation or that his entire epidermis has a minor defect in such incorporation which we are unable to detect, it is unlikely that his clinical signs of XP, including his numerous skin tumors, are etiologically related to a reduced rate of UV-induced ^{3}HTdR incorporation. In this regard it should be noted that while the severity and appearance of the clinical manifestations of XP in patient 4 were indistinguishable from those of patient 1, the difference in the ability of these patients' cells to perform UV-induced ^{3}HTdR incorporation is considerable. The clinical disease in patient 4, therefore, may have a basis other than that of most other XP patients. Alternatively, the as yet unknown etiologic defect in patient 4 may be present in all the other XP patients, and their DNA repair defect, reflected in an impaired rate of UV-induced ^{3}HTdR incorporation, may not be the cause of their clinical disease.

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